Cutting Edge: Mouse NAIP1 Detects the Type III Secretion System Needle Protein

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The NAIP/NLRC4 inflammasomes activate caspase-1 in response to bacterial type III secretion systems (T3SSs). Inadvertent injection of the T3SS rod protein and flagellin into the cytosol is detected through murine NAIP2 and NAIP5/6, respectively. In this study, we identify the agonist for the orphan murine NAIP1 receptor as the T3SS needle protein. NAIP1 is poorly expressed in resting mouse bone marrow–derived macrophages; however, priming with polyinosinic-polycytidylic acid induces it and confers needle protein sensitivity. Further, overexpression of NAIP1 in immortalized bone marrow–derived macrophages by retroviral transduction enabled needle detection. In contrast, peritoneal cavity marrows–derived macrophages express NAIP1 and respond to needle protein robustly, independent of priming. Human macrophages are known to express only one NAIP gene, which detects the needle protein, but not rod or flagellin. Thus, murine NAIP1 is functionally analogous to human NAIP. *The Journal of Immunology,* 2013, 191: 3986–3989.

There are many cytosolic-surveillance pathways, including the Nod-like receptors (NLRs). Several NLRs are inflammasomes that act as a platform to activate caspase-1, which carries out two major functions: processing of two inflammatory cytokines (pro–IL-1β and pro–IL-18) into their mature secreted forms and induction of a proinflammatory lytic cell death termed “pyroptosis.” NLRC4 is an inflammasome that primarily responds to infection by bacteria that use T3SS and T4SS. NLRC4 was first shown to respond to flagellin in the host cell cytosol (2, 3), which is inadvertently injected by the T3SS (4). However, NLRC4 responded to naturally flagellated *Shigella flexneri* (5), as well as *Salmonella typhimurium* and *Pseudomonas aeruginosa* flagellin mutants (6, 7). This flagellin-independent response was later attributed to detection of the T3SS rod protein (8), which presumably is also accidentally translocated into the host cytosol (9, 10). Interestingly, both flagellin and rod were detected through the same NLRC4 inflammasome (8). The detection of these two proteins through the same NLRC4 inflammasome was recently explained by the existence of distinct upstream NLRs in the NAIP family.

*C57BL/6 mice have four functional NAIP transcripts (NAIP1, NAIP2, NAIP5, and NAIP6), whereas only one NAIP gene has been described in humans (11). In mice, NAIP2 detects rod proteins, whereas NAIP5 and NAIP6 detect flagellins, and NAIP1 remains an orphan receptor (12, 13). Although the single human NAIP is highly homologous to mouse NAIP5, it does not activate NLRC4 in the presence of flagellin. Instead, human NAIP detects the needle protein, whereas flagellin and rod are not detected (13). In this study, we identify the T3SS needle protein as the agonist for the orphan mouse NAIP1, which is functionally homologous to human NAIP. This detection event was not observed previously because murine bone marrow–derived macrophages (BMMs) do not express sufficient levels of NAIP1 in the resting state.

**Materials and Methods**

**Tissue culture**

BMMs were prepared from the femurs of C57BL/6 mice by culturing with L cell–conditioned supernatants. Human U937 monocytes, obtained from the

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American Type Culture Collection, were cultured in RPMI 1640 containing 10% FBS and treated with 50 ng/ml PMA for 48 h to induce differentiation on plastic plates. Differentiated cells were lifted with PBS containing 1 mM EDTA and subcultured for assays. Resident peritoneal cavity (PerC) macrophages were harvested from naive mice using ice-cold PBS containing 1 mM EDTA. Peritoneal lavage fluid from two or three mice was pooled for each experiment.

**Retroviral transduction**

Cloning *Salmonella* PrgI and PrgJ into the pMXsIG retroviral system and the ensuing retroviral lethality screen were described elsewhere (8). Expression of bacterial proteins was assessed on day 2 posttransduction by flow cytometry. For *NAIP1* forced expression, Platinum-E cells (American Type Culture Collection) were transfected with MSCV2.2 retrovirus-based MSCV2.2 retrovirus-based plasmids. Purified proteins were transfected into macrophages using Polyfect (P2), as described previously (8). Lactate dehydrogenase activity in the supernatant was determined with the CytoTox 96 transfection reagent profect (P2), as described previously (8). Lactate dehydrogenase activity in the supernatant was determined with the CytoTox 96 transfection reagent profect (P2), as described previously (8).

**Protein transfections**

Cells were stimulated for 3–4 h with 50 ng/ml LPS (List Biological), unless indicated otherwise. PrgI-6XHis and PrgJ-6XHis were purified using Talon beads (Clontech). Purified proteins were transfected into macrophages using transfection reagent protect (P2), as described previously (8). Lactate dehydrogenase activity in the supernatant was determined with the CytoTox 96 assay (Promega), and IL-1β secretion was determined by ELISA (R&D Systems).

**Results and Discussion**

We wanted to verify that human macrophages respond to cytosolic needle and not rod protein (13). Cytosolic delivery of purified PrgI needle protein induced robust pyroptosis and IL-1β release from THP-1 and U937 cells, two differentiated human macrophage-like cell lines (Fig. 1A–D). However, there was no significant response to the PrgJ rod protein (Fig. 1A–D).

Interestingly, although we showed previously that BMMs cannot detect PrgI (2, 8), higher doses of cytosolic PrgI protein resulted in a weak response (Fig. 1E, 1F). To further confirm this finding, we used a retroviral lethality screen (8). BMMs from C57BL/6 or *Nlrc4Δ−/−* mice were retrovirally transduced with GFP only (empty vector), *prgI*-GFP, *prgJΔLRRs*-GFP, or *prgI*-IRES-GFP, and the surviving cells were analyzed by flow cytometry for GFP expression 2 d following transduction.

**Real-time quantitative PCR analysis**

Total RNA was isolated from indicated tissues, BMMs, or PerC macrophages using a RNeasy Mini Kit (QIAGEN), DNase treated (Promega RQ1), and reverse transcribed (Invitrogen), and quantitative TaqMan PCR was performed. The primers and probes used were described elsewhere (2). The amounts of mRNA analyzed were normalized to *GAPDH* or *Rps17*.

**Figure 1**

Human macrophages detect cytosolic PrgI, whereas mouse macrophages show a weak response. Human monocytic cell lines U937 (A, B) and Thp1 (C, D) were differentiated into macrophages with PMA, primed with 50 ng/ml LPS, and transfected with purified PrgI or PrgJ. Two hours posttransfection, cytotoxicity (A, C) and IL-1β secretion (B, D) were determined by lactate dehydrogenase release and ELISA, respectively. BMMs primed with LPS (50 ng/ml) for 4 h were transfected with 125 ng/well PrgI or PrgJ for 1 h, and cytotoxicity (E) or IL-1β secretion (F) was determined. (G and H) Cytotoxicity in response to cytosolic PrgI or PrgJ protein was determined at 2 h in unstimulated BMMs or BMMs primed with 6 μg/ml poly(I:C) for 2 d. Data are representative of two or three independent experiments.

**Figure 2**

Naip1 expression in BMMs, with or without stimulation with LPS or poly(I:C) from the innate immune database. (A) BMMs were left untreated or were primed with LPS or poly(I:C) for 23 h, and Real-time quantitative PCR was performed to assess Naip1 and Naip2 transcript levels relative to *Rps17*. (B) Cytotoxicity in response to cytosolic PrgI or PrgJ protein was determined at 2 h in unstimulated BMMs or BMMs primed with 6 μg/ml poly(I:C) for 2 d. Data are representative of two or three independent experiments.
Although GFP⁺ BMMs can be recovered from empty vector or \textit{prgJ}/\textit{LRRs}-GFP transduction, all \textit{prgJ-GFP}–transduced cells undergo pyroptotic cell death (Fig. 1G, 1H). Some \textit{prgJ-GFP} BMMs could be recovered but at somewhat reduced percentages (Fig. 1G, 1H). Together, these findings suggest a weak needle detection by BMMs. This reduction was dependent on \textit{Nlrc4} because \textit{Nlrc4−/−} BMMs showed a higher recovery of \textit{prgJ-GFP}–transduced cells (Fig. 1G, 1H), whereas empty vector was unaffected in the same experiment (47% in wild-type and 45% in \textit{Nlrc4−/−}) (8). These findings further establish that BMMs are weakly responsive to cytosolic \textit{PrgJ} in an \textit{Nlrc4}-dependent manner.

We hypothesized that \textit{PrgJ} was detected through the orphan NAIP1 receptor. But why is this response so inefficient? Activation of some inflammasomes occurs in a two-step process. For example, NLRP3 requires a priming step achieved by a TLR agonist, and AIM2 inflammasome response is enhanced by type I IFN priming (14, 15). In contrast, \textit{NLRC4} because \textit{NLRC4} is poorly expressed in BMMs as determined by quantitative PCR; however, it can be detected in some macrophage-rich tissues, including the spleen, small intestine, and colon (Fig. 4A, 4B). Interestingly, even under steady-state conditions, macrophages from the PerC of mice express substantial amounts of \textit{Naip1} (Fig. 4B). These findings are further corroborated in gene-expression profiling of immune cells by the ImmGen consortium (20). Therefore, we investigated whether PerC macrophages respond to \textit{PrgJ} without poly(I:C) priming. Indeed, \textit{PrgJ} induced robust IL-1β secretion from PerC macrophages compared with BMMs, and this was dependent upon \textit{Nlrc4} (Fig. 4B). We also confirmed that the flagellar hook (FlgE), which is structurally analogous to the needle, is not detected by PerC macrophages (Fig. 4B). Therefore, primary macrophages that express \textit{NAIP1} in detectable amounts are fully capable of sensing the needle protein without poly(I:C) priming. The physiological relevance of priming of the \textit{Naip1} pathway in vivo in other macrophage populations remains to be investigated.

Together, our studies show that detection of the T3SS needle in the cytosol requires \textit{NAIP1}. These results underscore the
importance of redundancy in the detection of T3SS. In mice, three distinct agonists, flagellin, rod, and needle, are targeted by four innate immune NAIP receptors, which converge upon a single NLRC4 inflammasome (Supplemental Fig. 1). Therefore, the ability to detect a variety of components of this important virulence structure ensures a thorough surveillance on the part of innate immune phagocytes.

Our findings reveal an unappreciated role for NAIP1 in the detection of T3SS needle and raise interesting questions about mouse versus human pathogen-recognition systems. Data published previously (13) and verified in this study indicate that humans seem to detect only the needle protein. Does the lack of rod and flagellin detection make humans more susceptible to Gram-negative pathogens that use T3SS? Future studies will examine the importance of flagellin, rod, and needle detection through NAIP5/6, NAIP2, and NAIP1, respectively.

Disclosures
The authors have no financial conflicts of interest.

References
Supplemental Figure 1.

A. BACTERIAL Cytosol

IM Inner Membrane
OM Outer Membrane

HOST cell cytosol

T3SS apparatus

Flagellar system

Periplasm

Plasma membrane

B. MOUSE HUMAN

Flagellin

NAIP5/6

Rod

NAIP1

Leucine-rich repeat (LRR)

CARD

BIR domain

Caspase-1

pro-IL1β
pro-IL-18

IL-1β
IL-18

Pyroptosis

Caspase-1

NLRC4

Vacuole

Needle

hNAIP

pro-IL1β
pro-IL-18

IL-1β
IL-18

Pyroptosis
Supplemental Figure 1. Models depicting the flagellar and the SPI1 secretion systems and activation of the NLRC4 inflammasome in mice and humans. (A) In the flagellar system, flagellin is transported through the hollow hook-basal body, and polymerizes to form filamentous flagella. In the T3SS SPI1 apparatus, rod protein lines the inside of the apparatus while needle protein polymerizes beyond the bacterial outer membrane and links with the pore complex inserted into the host cell membrane. b) Model shows the activation of NLRC4 inflammasome via distinct structural components of bacteria through NAIPs in mice and humans. In mice, NAIP2 and NAIP5/6 detect the cytosolic presence of rod and flagellins respectively. Here we describe that similar to accidental injection of rod and flagellins through the T3SS, needle protein monomers may also be exported into the host cytosol whereby they are detected by NAIP1. In humans, a single NAIP detect cytosolic needle protein. In both mice and humans, the NAIPs trigger NLRC4 oligomerization. NLRC4 then activates caspase-1, which processes IL-1β and IL-18 to their mature/secreted forms, as well as triggering pyroptotic cell death.